EXHIBIT B

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Indole Amine Deficiency in Blood and Cerebrospinal Fluid From Patients With Human Immunodeficiency Virus Infection

M. Larsson, L. Hagberg, G. Norkrans, and A. Forsman
Departments of Psychiatry III (M.L., A.F.) and Infectious Diseases (L.H., G.N.) University of Göteborg, Sweden

Twenty-four patients with human immunodeficiency virus (HIV) infection were investigated for possible changes in certain indole amine constituents in blood and cerebrospinal fluid (CSF). Albumin in serum was determined and used as a rough nutritional marker. Six of the 24 patients had acquired immunodeficiency syndrome AIDS, four had other clinical symptoms of HIV infection, and 14 had no apparent symptoms. The HIV-seropositive patients had significantly decreased tryptophan values; their blood concentrations were 28% lower and their CSF concentrations 30% lower than corresponding values in 14 healthy controls. The blood concentrations of 5-hydroxytryptamine (5-HT) were 50% lower, and the platelet content of 5-HT was 36% lower in HIV-infected individuals than in the control group. The most pronounced changes were invariably seen in the six cases with AIDS and in patients with a low number of $CD4^+$ cells. No significant difference between controls and HIV-seropositive patients was detected in the mean CSF concentrations of 5-hydroxyindoleacetic acid (5-HIAA), although these levels were markedly reduced in four of the HIV patients. Neither was any significant difference seen between patients and controls in the serum concentrations of albumin.

Key words: HIV, AIDS, tryptophan, 5-HT, 5-HIAA, blood, CSF

INTRODUCTION

Symptoms of the central nervous system (CNS), diarrhea, and acquired immune deficiency syndrome (AIDS) cachexia ("slim disease") are common in the advanced stages of acquired immunodeficiency syndrome but may also appear as primary manifestations of HIV infection in man (Navia et al., 1986; Serwadda et al., 1985). These symptoms are often seen in the absence of any concurrent illness other than human immunodeficiency virus (HIV) infection to explain the findings.

$CD4^+$ helper/inducer T-lymphocytes are the primary target for HIV, but the virus has also been detected in brain tissue macrophages (Koenig et al., 1986), in cerebrospinal fluid (CSF), (Ho et al., 1985; Åsjö et al., 1986; Chiodi et al., 1988), and in gastrointestinal epithelium (Nelson et al., 1988). Because, however, only a very small fraction of all $CD4^+$ cells appear to be productively infected with HIV, it is unlikely that direct viral destruction is a pathogenic mechanism for all symptoms associated with the disease (Ho et al., 1987). Thus, the evidence suggests that secondary effects of the infection cause some of the symptoms.

Assuming that HIV-induced changes in the brain and in the gastrointestinal tract might be reflected by abnormal concentrations of blood and CSF constituents associated with diseases in these tissues, we have analyzed some indole amine compounds in HIV patients and healthy controls. The blood concentrations of tryptophan and 5-hydroxytryptamine (5-HT) and the CSF concentrations of tryptophan and 5-hydroxyindoleacetic acid (5-HIAA) were determined. The number of platelets was studied in relation to the blood levels of 5-HT, and the number of helper cells was assessed in blood. Serum concentrations of albumin were determined for use as a rough marker reflecting the nutritional status of the patients.

MATERIAL AND METHODS

Subjects

The study involved 24 HIV-seropositive patients, 21 men and three women, aged 19–69 years (mean age 35 years), living in the city of Göteborg (Sweden). Six had AIDS, as defined in accordance with the surveillance criteria of the Center for Disease Control (CDC). Other clinical symptoms of HIV infection were diagnosed in

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Address reprint requests to Margareta Larsson, M.D., Department of Psychiatry III, Lillhagen Hospital, 422 03 Hisings Backa, Sweden.

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TABLE I. Clinical Data in 24 Patients With HIV Infection

	Sex/age	Years with HIV	Risk factor	Somatic complications	Psychiatric complications	Number of helper cells[a]
Patients with AIDS						
Case 1	M/31	?	Homosexuality	Pneumocystis	Dementia	<0.02
Case 2	M/40	?	Homosexuality	Pneumocystis, Kaposi's sarcoma		0.02
Case 3	M/37	?	Homosexuality	Pneumocystis	Dementia	0.02
Case 4	M/26	?	Homosexuality	Pneumocystis		0.02
Case 5	M/45	>4	Homosexuality	Pneumocystis		
Case 6	M/58	1.5	Homosexuality	Lymphoma		0.29
Symptomatic patients						
Case 7	M/69	1	Transfusion	GB, myeloplastic disorder		0.06
Case 8	M/49	0.5–1	Homosexuality	LAS		
Case 9	M/26	?	Homosexuality	LAS		0.19
Case 10	M/33	2	Homosexuality	ARC (diarrhea)		0.53
Asymptomatic patients						
Case 11	M/21	>4	Homosexuality		Dementia, psychosis	0.32
Case 12	M/28	0.5	Homosexuality			0.56
Case 13	F/37	1.5	Transfusion			0.38
Case 14[b]	M/25	3				0.46
Case 15	M/25	2–3	Homosexuality			0.71
Case 16	M/24	?	Homosexuality	Condyloma		1.29
Case 17	M/33	?	Homosexuality	Nonpurulent coxarthritis		0.37
Case 18	M/31	?	Homosexuality			2.10
Case 19	M/40	?	Homosexuality			0.46
Case 20[b]	F/28	5				0.12
Case 21	M/47	1	Homosexuality			0.56
Case 22	M/39	>2	Homosexuality		Dyslexia, dysgraphia	0.34
Case 23	F/19	?	Drug addiction			0.38
Case 24	M/25	0.5	Homosexuality	Aseptic meningitis		0.90

[a]Total number of helper cells ($\times 10^9$/liter).
[b]Heterosexual transmission.

four patients: two with lymphadenopathy syndrome (LAS), one with Guillain Barré's syndrome (GB), and one with AIDS-related complex (ARC) *(symptomatic* cases). Fourteen patients were seropositive for HIV antibodies but showed no somatic symptoms of HIV infection (*asymptomatic* cases). Dementia or other neuropsychiatric abnormalities were seen in two AIDS patients and in two otherwise somatically healthy cases. Clinical data of the patients are given in Tables I and II. A group of 14 healthy and drug-free volunteers, aged 23–58 years (mean age 33), was used for comparative analyses.

CSF

CSF and blood samples were collected from patients and controls in the morning before breakfast. The subjects were instructed to rest, but they were not confined strictly to bed during the night before sampling. With the subject in a lateral recumbent position, the lumbar puncture was made between L IV and L V, and 22 ml of CSF was obtained from each person. In accordance with standardized procedures, CSF flow was divided into fractions for use in various analyses; concentrations of 5-HIAA and tryptophan were determined in the 13:th ml fraction. Samples were frozen at −70°C immediately after collection and stored until analysed.

An ethylenediaminetetraacetic acid (EDTA) blood sample was drawn from each individual in connection with the CSF sampling. Immediately after sampling for duplicate determinations of tryptophan and 5-HT, 2 × 2 ml blood was transferred to new glass tubes containing 6 mg ascorbic acid. All samples were frozen and stored at −20°C until they were analysed. Platelets were counted in fresh blood from each sample for estimation of 5-HT content per platelet.

Analytical Techniques

The indole amine analyses in blood and CSF were performed by means of high-performance liquid chromatography (HPLC) technique with fluorescence detection. The Perkin-Elmer LS 4 detector was set to Ex = 280 nm and EM = 345 nm, with slits of 10 nm and 20 nm, respectively. The column was a 10 μm μBondapack C_{18} (3.9 mm × 30 cm) from Waters Associated Inc, set at a flow rate of 1.5 ml/min. The mobile phase consisted of a 0.01 M citric acid monohydrate/tri-sodium citrate 2-hydrate buffer at pH = 4.1. and CH_2OH (92:8). Imme-

TABLE II. Pharmacological Treatment in 24 Patients With HIV Infection

Case number	Substances
1	Amphotericinum B
2	Aciclovirum + pyrimethaminum + calcii folinas + amphotericinum B
3	Aciclovirum + pyrimethaminum + calcii folinas + amphotericinum B
4	No medication
5	Trimethoprimum/sulfamethoxazolum + flunitrazepanum
6	No medication
7	Dextropropoxiphenum + paracetamolum/chlormezanonum + nitrazepanum
8	No medication
9	Metronidazolum + paramomycinum
10	No medication
11	Thioridazine prescribed but probably not taken
12–16	No medication
17	Cloxacillinatrium + dextropropoxiphenum
18–22	No medication
23	Diazepanum + alimemazinum
24	No medication

diately after thawing, the blood samples were precipitated with 200 μl ascorbic acid (10%), 200 μl EDTA (10%), and 8 ml perchloric acid (0.4 M). After centrifugation, 25 μl of the clear supernatant was injected into the described HPLC system for assay of 5-HT and tryptophan. From each thawed CSF sample, 50 μl was directly injected into the same HPLC system for assay of tryptophan and 5-HIAA (Larsson et al., 1988a).

HIV antibodies were tested by enzyme-linked immunosorbent assay (Behring and Wellcome) and confirmed by Western blot. Electroimmunoassay was used for determination of the serum concentrations of albumin (Laurell, 1972).

The number of T-helper-inducer cells ($CD4^+$) was measured by means of leu-3 monoclonal antibodies according to standard methods.

Statistics

Statistical evaluations of differences between groups of values were performed by means of a *t*-test.

RESULTS

Tryptophan in Blood and CSF

Blood and CSF concentrations of tryptophan in the HIV patients and healthy subjects are presented in Table III. Blood tryptophan levels below the lowest value in the group of healthy subjects were seen in seven asymptomatic patients, two patients with LAS, one with GB, and five with AIDS. CSF concentrations of tryptophan below the lowest value in the healthy group were found in 11 of the 14 asymptomatic patients, in both patients with LAS, in the patient with ARC, and in all patients with AIDS (Fig. 1).

The tryptophan levels were significantly lower in the asymptomatic patients than in the healthy subjects ($P<0.01$ for blood as well as for CSF concentrations) and still further reduced in the patients with AIDS ($P<0.001$ for blood as well as for CSF concentrations (Fig. 1). The lowest blood tryptophan value, 2.8 μg/ml, was seen in two patients suffering from dementia (cases 1 and 3).

The blood/CSF tryptophan concentration ratio in the healthy subjects ($n = 14$) varied between 14.6 and 23.0 (mean 18.0). Ratios above 23.0 were noted in one patient with AIDS and in five other cases. The range among the other 18 patients was 9.2–23.0 (mean 16.8).

5-HIAA in CSF

No significant change in the CSF 5-HIAA concentrations was observed among the HIV aptients (Table III), but in two asymptomatic patients, one symptomatic patient, and one AIDS patient, i.e., in 17% of the cases, 5-HIAA concentrations were reduced as compared with healthy subjects.

Platelets and 5-HT

The number of platelets was significantly lower in patients with HIV infection than in the healthy subjects (Fig. 2, Table III). Five asymptomatic patients, one with GB, and two with AIDS had platelet counts below the lowest value in the control group.

Blood concentrations of 5-HT in the HIV patients were, in mean, 50% lower than in the healthy subjects

Table III. Laboratory Findings in Healthy Subjects and Patients With HIV Infection*

	Healthy subjects (n = 14)		HIV patients (n = 24)		Reduction in mean values (%)	Significance of difference
	Mean	Range	mean	range		
Number of platelets ($\times 10^9$/liter)	238	140–375	184	32–287	23	$P < 0.02$
Blood concentration of 5-HT (ng/ml)	153	97–374	76	15–175	50	$P < 0.001$
Platelet-bound 5-HT (ng/10^9 platelets)	676	416–1257	430	64–1018	36	$P < 0.01$
Blood concentration of tryptophan (μg/ml)	8.1	6.0–11.1	5.8	2.8–9.2	28	$P < 0.001$
CSF concentration of tryptophan (ng/ml)	445	371–513	310	30–531	30	$P < 0.001$
CSF concentration of 5-HIAA (ng/ml)	23	13–42	25	10–47		n.s.
Serum concentration of albumin (g/liter)	41.5	37–49	40.3	29–51	2.9	n.s.

*n.s. = not significant.

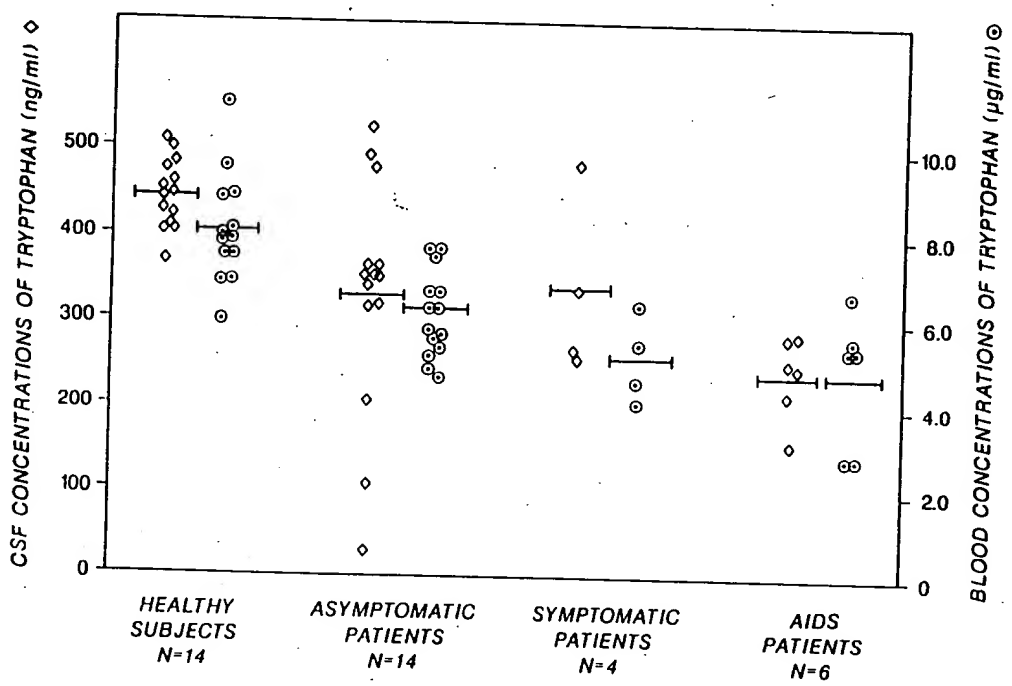

Fig. 1. Blood and CSF concentrations of tryptophan in healthy subjects and in patients with different degrees of HIV infection. Mean value is indicated in each group.

(Table III). The ratio between the blood concentrations of 5-HT and the number of platelets, i.e., the platelet content of 5-HT, was found to be reduced in the HIV patients (Table III). Levels of platelet-bound 5-HT below the lowest value in the control group were found in six asymptomatic patients, in two symptomatic patients (1 with LAS, 1 with GB), and in five of the six AIDS patients. The mean 5-HT content of the platelets was decreased with 30% in the asymptomatic patients ($P<0.02$) and with 56% in the AIDS patients ($P<0.01$), as compared with the corresponding value in the control group. This decrease in the mean platelet-bound 5-HT levels with increasing severity of HIV infection is illustrated in Figure 2.

Among the six patients with AIDS, the lowest levels of 5-HT per 10^9 platelets were seen in the three patients who also had the highest number of platelets (Fig. 2). A normal number of platelets, in combination with decreased amounts of platelet-bound 5-HT, was also observed in one patient with LAS and in four asymptomatic patients. No similar finding was made in the group of healthy subjects.

$CD4^+$ Cells and Tryptophan

The total number of $CD4^+$ cells was decreased in those patients having a severe degree of HIV infection (Table I). A logarithmic regression model showed a positive correlation between the total number of helper cells (x) and the blood concentration of tryptophan (y) in the

creased severity of the infection. Also, the lowest tryptophan levels were found among the patients with AIDS.

The HIV patients also had reduced CSF concentrations of tryptophan as compared with healthy subjects. The blood/CSF concentration gradient of tryptophan, however, was essentially unchanged in all but two of the HIV patients (cases 11 and 22, who also had signs of organic brain disease (Table I)). Three other symptomatic patients and one AIDS patient had slightly elevated ratios. The fact that the ratios in most cases were unchanged indicated that the low CSF tryptophan concentrations might be due to low blood concentrations of the amino acid rather than to degradation inside the CNS. In addition, the majority of the HIV patients had CSF concentrations of 5-HIAA within the range of values among the healthy subjects, indicating a functional synthesis of the neurotransmitter. This finding does not, however, exclude possible changes in the release and turnover of the transmitter or in the postsynaptic receptor functions. Findings in the four patients with decreased CSF concentrations of 5-HIAA could indicate an affected metabolic pathway of serotonin in the CNS in these cases.

The number of platelets, as well as the concentrations of 5-HT in blood, was reduced in patients with HIV infection. Because the blood-5-HT to a great extent is bound to the platelets, which store the amine but do not synthesize it, concentrations of 5-HT were related to the number of platelets. Also, the platelet content of 5-HT was reduced in patients with HIV infection when compared with healthy subjects. The most pronounced reductions in platelet-bound 5-HT were seen in AIDS patients with a normal number of platelets. As the difference could not be ascribed to any known mechanism associated with the medication in the patient group (Table II), the low platelet content of 5-HT could be caused by defective platelets (Stricker et al., 1985; Beardsley et al., 1984), or they might result from a defective tryptophan metabolism.

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